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**14. ABSTRACT**
We used a novel method to discover tumor antigens with the intent to develop a diagnostic assay for lung cancer. We isolated intratumoral B lymphocytes (ITLs), cloned their immunoglobulin genes, and used the expressed antibodies to identify tumor antigens. All of the antibodies produced from ITLs in our study were polyreactive. One antibody that preferentially recognized tropomyosin 4 was identified; this antibody was confirmed in the serum of the same cancer patient, and in a small pilot study, the tropomyosin 4 antibody was found to be specific for the diagnosis of lung cancer, but was not very sensitive (20%). We are currently modifying our approach to identify those ITLs that show evidence of clonal expansion prior to the cloning and expression of recombinant antibodies. This will increase the likelihood that the recombinant antibodies produced are the result of proliferation and differentiation in response to tumor antigens. This will allow us to concentrate our cloning, antigen identification, and validation efforts on those antibodies that are more likely to demonstrate high affinity monoreactivity against tumor antigens. Isolation of single ITLs and cloning of their immunoglobulin genes has the potential to be a useful approach for identifying diagnostic biomarkers or novel molecular targets.

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## Table of Contents

**Page**

## Introduction

The aim of this research is to develop a blood test for autoantibodies that will determine whether a lung nodule discovered upon X-ray or CT is cancer. Our method is to first identify autoantibodies made by antigen stimulated, intratumoral B lymphocytes in patients with non-small cell lung cancer (NSCLC). These antibodies are then used to identify the stimulating antigens, and the antigens are used to formulate a diagnostic blood test for lung cancer.

Intratumoral B lymphocytes are associated with many types of cancer including NSCLC (1-3), breast cancer (4,5), and ovarian cancer (6). Analysis of B cells from tumors and metastatic lymph nodes found hypermutated antibody genes and expansion of class switched memory cells and plasmablasts in patients with bladder cancer (7). In addition, the presence of intratumoral germinal centers (GCs) have been reported in NSCLC (8,9), breast cancer (10-13), colon cancer (14), and germ cell cancers (15). In NSCLC, GCs are associated with early stage over late stage disease, suggesting a humoral immune response may hold these tumors in check (9).

Further characterization of antigen stimulated intratumoral B cells and identification of tumor antigens might be exploited for diagnostic or therapeutic purposes. The research described in this report focuses on detection of autoantibodies produced by intratumoral B cells for cancer diagnosis. We have isolated single intratumoral B cells from a lung tumor, cloned their heavy and light chain genes, expressed their immunoglobulins, and identified a stimulating tumor antigen. We then identified antibodies to tropomyosin 4 (TPM4) expressed by an intratumoral B cell of one lung cancer patient. We confirmed the presence of the antibody in this patient's serum, and then performed a pilot study to determine if the serum autoantibody could be used as a diagnostic biomarker for lung cancer.

## Body

## Materials and Methods

*Patients*

The current study was approved by our Institutional Review Board and all patients signed an informed consent prior to enrollment in the study. Intratumoral lymphocytes (ITLs) used in this study were isolated from freshly resected stage I lung adenocarcinoma tissue from a patient undergoing surgery at Duke University Medical Center. For the ELISA study, we isolated serum from blood collected in red-top Vacutainer tubes from 15 patients with a new diagnosis of NSCLC and 15 without evidence of cancer. The NSCLC group included 7 patients with stage I disease, 2 with stage II, 4 with stage III, and 2 with stage IV. Sera were stored at -80°C until use.

*Cell lines*

Human embryonic kidney 293T cells were maintained in DMEM containing 10% (v/v) heat-inactivated FBS and 50 µg/ml gentamicin. Lung carcinoma H460 cells (ATCC) were maintained in RPMI-1640 medium containing 10% (v/v) FBS. Media and supplements were obtained from Gibco (Invitrogen, Carlsbad, CA) and cells were maintained in a humidified atmosphere at 37°C and 5% $CO_2$.

*B Cell Isolation and FACS*

We isolated ITLs from lung tumor specimens by first placing the tissue in a Petri dish containing RPMI-1640 medium supplemented with 20 mM HEPES (RPMI/HEPES) and teasing the tissue into very small fragments with an 18 gauge needle. We then filtered the tissue fragment suspension sequentially through 100 µm and 40 µm pore-size nylon membranes and pelleted the cells by centrifugation for 10 min at 400 x g at 18°C. We resuspended the cell pellet in 2 ml RPMI/HEPES and isolated the lymphocytes over a Ficoll-Paque Plus (GE

Healthcare, Uppsala, Sweden) cushion according to the manufacturer's instructions. We resuspended the final lymphocyte pellet in Bambanker (Wako Chemicals, Richmond, VA) cell freezing medium and stored the cells at -80°C until FACS.

For sorting of memory B cells and plasma cells, we first thawed the ITLs and washed them in PBS. We then stained the cells with Aqua vital dye (Invitrogen, Carlsbad, CA) and a combination of the following anti-human antibodies: CD3 phycoerythrin (PE)-Cy5, CD14 PE-Cy5, CD16 PE-Cy5, CD235a PE-Cy5, CD45 PE-Texas Red, CD19 allophycocyanin (APC)-Cy7, CD27 PE-Cy7, CD38 APC-Cy5.5, immunoglobulin M (IgM) FITC, and IgD PE (BD Biosciences, Mountain View, CA; Beckman Coulter, and Invitrogen). During the sort, we used forward- versus side-scatter gating to select for lymphocytes, and geometric gates to eliminate doublet events. We gated B cells as $CD45^+$, $CD3^-$, $CD14^-$, $CD16^-$, $CD235a^-$, and $CD19^+$; total memory B cells were further identified as IgD negative ($IgD^-$). B cells were sorted individually into 96-well PCR plates containing ice-cold PBS, DTT, and RNAsin (Promega, Madison, WI) and stored at -80°C until further processing. We performed FACS on a BD FACSAria (BD Biosciences, San Jose, CA) and analyzed the data with FlowJo (Tree Star, Ashland, OR).

*Antibody Cloning and Expression*

We amplified the immunoglobulin heavy and light chain variable (VH and VL) gene regions from individual sorted cells by RT-PCR using a method first described by Tiller et al. (16) and modified by Liao et al. (17). Briefly, we reverse transcribed total RNA from single cells using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and human IgG, IgM, IgD, IgA1, IgA2, Ig kappa (κ) and Ig lambda (λ) constant region primers; the sequences of these primers are in Supplementary Table 1 of reference (17). We then used the resulting cDNA in nested PCR reactions to amplify the VH, Vκ and Vλ variable regions as previously described (17). Using tag sequences that were part of the second-round nested PCR primers, we cloned the VH and VL genes into human Ig(γ) and Ig(λ) pcDNA3.1(+) (Invitrogen) expression vectors and sequenced the inserts to confirm identity with the original PCR product.

*Recombinant Antibody Purification*

For production of recombinant antibodies, we co-transfected 293T cells with pairs of pcDNA3.1(+) plasmids containing VH and VL genes. Each plasmid pair contained VH and VL genes obtained from a single sorted memory B cell or plasma cell. After 4 days in Pro293a-CDM serum-free medium (Lonza, Walkersville, MD) containing 1X GlutaMAX and 50 μg/ml gentamicin, we collected the culture medium containing the recombinant antibodies, concentrated it using Centricon Plus-70 30K MWCO centrifugal concentrators (Millipore, Billerica, MA), and purified each recombinant antibody using Protein G Agarose (Pierce, Thermo Scientific, Rockford, IL).

*Immunoblotting*

We tested recombinant antibodies for reactivity against H460 lung carcinoma cell lysate proteins by immunoblot. Proteins were separated by 1D-PAGE on a gel with a single preparative well, blotted to polyvinylidene fluoride membrane (PVDF, Millipore), and the PVDF inserted into a Surf-Blot apparatus (Idea Scientific Co., Minneapolis, MN) that creates individual channels that permits multiple different primary antibodies to be run simultaneously. We allowed the primary antibodies to interact with the blot for 2 h at room temperature, washed the membrane, and detected bound antibody with goat anti-human IgGγ chain-HRP conjugate. This was followed by incubation in chemiluminescent substrate and x-ray film exposure.

*Antigen Identification*

We identified the protein antigens responsible for immunoreactive bands in 1D-PAGE immunoblots by first probing 2D-PAGE blots of H460 lysate proteins with recombinant antibodies as described above. We then used the location of the immunoreactive spot as a guide to locate the protein of interest on a duplicate Coomassie-stained 2-D PAGE gel of the same lysate sample. Proteins in the stained spot were identified at

the Duke Proteomics Facility by in-gel tryptic digestion, MALDI-TOF MS peptide fingerprinting, and nano-LC MS/MS.

We carried out 2-D PAGE by loading the protein sample (125 µg of H460 lysate protein in 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM DTT, 0.2% (w/v) Bio-Lyte 3-10 ampholytes, and bromophenol blue (trace)) by passive rehydration into 7 cm, pH 3-10 NL Ready-Strip IPG strips (Bio-Rad, Hercules, CA). Isoelectric focusing was carried out at 4,000 V for a total of 10,000 V-h using a Protean IEF Cell (Bio-Rad). We then incubated the IPG strips sequentially in equilibration buffers consisting of 1X NuPAGE LDS sample buffer (Invitrogen) containing 50 mM DTT or 125 mM iodoacetamide for 15 min each. We carried out second dimension separation by SDS-PAGE on 4-12% NuPAGE Bis-Tris ZOOM gels (Invitrogen). Gels to be stained were incubated in SimplyBlue SafeStain (Invitrogen) for 1 h followed by destaining with water. Blotting to PVDF was carried out as for 1D-PAGE immunoblotting.

*TPM4 ELISA*

We evaluated the presence of antibodies against TPM4 in sera from patients with or without lung cancer by ELISA. Recombinant human TPM4 containing a 6x-His tag (Fitzgerald Industries, Acton, MA), was immobilized for 1 h at room temperature in the wells of His-Select nickel coated 96-well plates (Sigma-Aldrich, St. Louis, MO) that had been pre-blocked with IgG-free BSA. After removing unbound proteins by washing in PBS containing 0.1% (v/v) Tween-20 (PBST), we added serum that had been diluted 1:50 in PBST to the wells and incubated the plates for 1 h at room temperature. After washing the wells, bound antibody was detected with anti-human IgGγ chain-HRP (1:1000) followed by development in ABTS and hydrogen peroxide. Absorbance was determined at 405 nm in a plate reader. The final absorbance values were calculated by subtracting the absorbance of wells without TPM4 from those with TPM4. The p-value was calculated according to Student's t-test.

## Results

*Antibody Cloning and Expression, Purification, and Confirmation of Reactivity with Tumor Antigens*

The overall work flow for the current study is shown in Fig. 1. We performed FACS on 2.6 x $10^6$ total ITLs isolated from a 0.133 g (wet weight) piece of freshly resected early stage lung adenocarcinoma. Total B cells, which constituted 17.6% of the ITL population, were further delineated into transitional, naïve, and memory B cells (Fig. 2). Total B cells (CD19+) or memory B cells (CD19+ sIgDneg) were sorted into individual wells of three 96-well PCR plates. Single cell RT-PCR yielded 8 heavy chain gene products from 8 wells; in three wells two different light chains were isolated. One light chain gene was not further characterized; two isolated light chain genes from different wells were identical at the amino acid level and only one of these was produced as a plasmid for pairing with the appropriate heavy chain genes. The characteristics of the 8 distinct heavy chain genes and 9 light chain genes are given in Table 1. Heavy chain genes associated with two light chains were transfected in heavy-light pairs to produce both possible antibodies for further characterization and were assigned individual recombinant antibody designations (Table 1).

We cloned the isolated VH and VL Ig genes described above into the mammalian expression vector pcDNA3.1(+), resulting in 8 plasmids containing VH genes and 9 plasmids containing VL genes. For expression of recombinant antibodies, we co-transfected appropriately matched pairs of VH and VL immunoglobulin gene-containing plasmids into 293T cells and harvested the recombinant antibody-containing cell culture supernatant after 96 hours of culture. As shown in Table 1, VH plasmids pH006251 and pH006247 were each paired with two different VL plasmids, and VL plasmid pK004629 was paired with two different VH plasmids, yielding a total of ten pairs of plasmids for transfection into 293T cells. The resulting ten recombinant antibodies were purified from the cell culture supernatants using protein G agarose. An image of a silver-stained polyacrylamide gel containing all 10 purified recombinant antibodies is shown in Fig. 3. Both heavy Ig chains (bands above the 55 kDa marker) and light Ig chains (bands between the 25 and 35 kDa markers) are visible in each lane.

Since these recombinant antibodies had been cloned from tumor-infiltrating B cells, we were interested in examining their immunoreactivity against lung cancer proteins. We did this by probing blots of proteins from the lung carcinoma cell line H460 with each recombinant antibody. One-dimensional immunoblots showed the recombinant antibodies to be immunoreactive against multiple proteins, suggesting polyreactivity (18). Two-dimensional immunoblots confirmed this but also showed that one antibody, T009, was exceptionally immunoreactive against a protein of approximately 35 kDa (data not shown). This raised the possibility that T009 might exhibit a functional monoreactivity toward this antigen in vivo, and might even exhibit tumor selectivity. Hence, we set out to determine the identity of the protein responsible for this immunoreactivity.

*Antigen Identification and Confirmation*

In order to identify the ~35 kDa immunoreactive protein seen in immunoblots, we used T009 to perform an additional 2D immunoblot of H460 lysate and then aligned the resulting immunoreactive spot with a spot on a duplicate gel stained with Coomassie (Fig. 4). The spot was cut out of the stained gel and submitted to the Duke Proteomics Facility for identification by nano-LC MS/MS. This analysis identified tropomyosin alpha-4 (TPM4) as the putative immunoreactive protein.

To determine if recombinant antibody T009 indeed recognized TPM4, we performed an immunoblot analysis of purified recombinant TPM4 protein (Fig. 5A). As shown in the figure, T009 is clearly immunoreactive against TPM4. We also confirmed that serum antibodies, from the same patient from whose tumor lymphocytes the TPM antibody genes were cloned, recognize purified TPM4 protein (Fig. 5B).

TPM4 is a member of the tropomyosin family of actin binding proteins. Anaplastic lymphoma kinase (ALK) is a receptor-type protein tyrosine kinase that is rendered oncogenic as a result of its fusion to TPM4 in inflammatory myofibroblastic tumor (19). We investigated whether the antibody to TPM4 may have arisen due to the presence of such a fusion event by performing fluorescence in situ hybridization analysis on the tumor tissue that provided the B cells from which recombinant antibody T009 was cloned. However, no evidence for a translocation involving TPM4 was found in this tissue (data not shown).

Since it was possible that anti-TPM4 antibodies in this patient's serum arose due to a tumor-specific immune response, we were interested in comparing the prevalence of the antibodies in serum from lung cancer patients and individuals without cancer. We surveyed the serum from 26 patients with lung cancer and 21 without lung cancer using a direct ELISA developed in our laboratory. As shown in Fig. 6, antibodies against TPM4 were detected in serum from patients both with and without lung cancer. Approximately 42% (11/26) of patients with lung cancer had the antibody compared to 29% (6/21) without cancer. Although these differences are not statistically significant ($p= 0.118$), by ROC analysis the sera exhibiting the 5 highest ELISA values (> 47.74) were all from patients with lung cancer (specificity - 100%, sensitivity - 20%).

## Discussion

Tumor growth reflects a dynamic, multidimensional relationship between cancer cells and host cells, including stromal, endothelial, and immune cells (20,21). Tumors can be infiltrated with many types of lymphocytes, some that may foster and others that may inhibit tumor growth and progression (22). GCs, normally found in secondary lymphoid organs such as the spleen and lymph nodes, can also develop in tumors. GCs are well-defined loci of B cells, T cells and dendritic cells in which B cells proliferate and undergo differentiation, somatic hypermutation and affinity maturation (23). The presence of GCs in tumors is consistent with an in situ immune response to tumor antigens. We aimed to develop a method to identify the targets of antibodies produced by intratumoral B cells. This would allow us to explore the possibility that tumor antigens targeted by these intratumoral B cells may be relevant therapeutic targets or that the antibodies they produce may be diagnostic biomarkers.

Many tumor-associated antigens have been identified in the serum by techniques such as SEREX (24). However, serum antibodies have unknown etiology; e.g., they may be pre-existing autoantibodies that weakly recognize tumor antigens. Nevertheless, it is clear that some intratumoral B cells express antibodies against tumor antigens. Punt et al. (25) isolated and cultured tumor-infiltrating B cells and tested the IgG from culture supernatants: the IgG from 25 of 36 B cells from tumor cell suspensions showed reactivity with autologous tumor targets. Ten of thirteen IgGs reacted with allogeneic tumor targets of the same histological diagnosis but no reactivity was found with tumor targets of a different histology. Antibodies produced by B cells within a tumor may be more likely to be directed against relevant targets than those produced by peripheral B cells, particularly if they demonstrate antigenic stimulation, class switching, affinity maturation and somatic hypermutation. To date, very few targets of antibodies produced by B cells located within a tumor have been directly identified. Kotlan et al. (12) constructed an scFv library from IgG variable chain genes derived from human medullary breast carcinoma-infiltrating B lymphocytes and isolated an scFv that binds to gangliosides, which had been previously identified as tumor-associated antigens. Yasuda et al. (26) engrafted human lung tumor tissue in SCID mice and identified antibodies to mutated p53 in the mouse serum. These antibodies had presumably originated from B cells in the engrafted tumor tissue.

Here, we have used methodology originally developed to study the role of antigen in the progression of chronic lymphocytic leukemia (27) and later modified by researchers in the HIV field to permit the cloning of human antibodies from antigen-secreting B cells (17,28). Our study represents the first attempt to adapt this methodology to characterize single B cells isolated from a solid tumor and to identify their stimulating antigens. In this approach, immunoglobulin VH and VL genes are amplified by RT-PCR from single sorted B cells and separately inserted adjacent to sequences encoding constant regions in the expression vector pcDNA3.1(+). Transient transfection of the resulting heavy chain and light chain plasmids into human 293T cells yields mature antibody, which is then purified. The purified antibody is used to probe two dimensional immunoblots of lung tumor cell lysates. The protein is excised from a corresponding stained gel and identified by peptide fingerprint analysis and sequencing by tandem mass spectrometry. Antibody reactivity with purified target protein is then confirmed.

In the current study, we focused on lung cancer where the majority of patients have advanced stage disease at presentation when cure is unlikely. Hence, new therapeutic and diagnostic strategies are clearly needed (29). This approach provided a unique opportunity to take cues from the host immune response to the tumors and possibly discover new therapeutic or diagnostic targets. From this pilot study several interesting observations were made:

First, we showed that the ITLs demonstrated class switching as only one of the mAbs tested was originally IgM; the remaining mAbs were IgG1, IgG3, and IgA1 (Table 1). The mean heavy chain mutation frequency was 4.00% ± 1.61% (range 1.72-6.16%), a mutation frequency typical of antibodies isolated from vaccine recipients (30,31). None of the recovered mAbs were clonally related to each other, and a range of VH genes were used (Table 1). In addition, heavy chain complementarity determining region 3 (HCDR3) loops ranged from 9-25 amino acids (median 13) with no single length predominating. These data are consistent with an antigen-driven maturation occurring in GCs within this tumor, although recruitment of preexisting memory B cells to the tumor cannot be excluded.

Second, all of the antibodies produced from ITLs in our study were polyreactive. Polyreactive antibodies are antibodies of low affinity (Kd of $10^{-3}$-$10^{-7}$ M) that react with a variety of totally unrelated antigens (18). In newborns, 50% of cord B cells express polyreactive antibodies (32) and in adults, 15-20% of peripheral B cells express polyreactive antibodies (18). Interestingly, polyreactive antibodies in the circulation appear to have broad antimicrobial properties (33). It is intriguing to speculate that polyreactive antibodies in tumors may, in the aggregate, have antitumor properties. Polyreactive antibodies resemble germline antibodies, and thus, a more fruitful approach to identifying monospecific antibodies expressed by B cells in tumors might be to focus

on those cells that have undergone affinity maturation and clonal expansion.

Third, we identified an antibody to TPM4 that was present in both cancer and non-cancer sera, although the higher levels were associated with the sera of cancer patients. TPM4 is an intracellular protein that interacts with the cytoskeleton so it is not apparent how an antibody was generated against it; possibilities include that it was released from dying cells or it was presented on the cell membrane as part of an aberrant gene fusion event. However, we found no evidence that TPM4 antibody T0009 recognized a fusion protein. This result, and the fact that the antibody is found in non-cancer sera makes it more likely that TPM4 is antigenic upon release from necrotic or apoptotic cells (34).

**Key Research Accomplishments**

- Adapted antibody cloning methodology from the HIV field for use in cancer.
- Developed platform for tumor antigen identification using antibodies cloned from ITLs.
- With refinement, this methodology should be useful in identifying tumor associated antibodies for diagnostics and tumor associated antigens for therapeutics.

**Reportable Outcomes**

Manuscript in preparation

**Conclusions**

Although adapting methodology from the HIV field to the study of cancer immunology has the potential to be very useful, the current study resulted in identifying predominately polyreactive antibodies, one of which may have some relationship to the host response to lung cancer. Future studies will focus on identifying those ITLs that show evidence of clonal expansion prior to the cloning and expression of recombinant antibodies. This will increase the likelihood that the recombinant antibodies produced are the result of proliferation and differentiation in response to tumor antigens. This will allow us to concentrate our cloning, antigen identification, and validation efforts on those antibodies that are more likely to demonstrate high affinity monoreactivity against tumor antigens.

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## Supporting Data

| recAb designation | sorting gate | original isotype | $V_H$ plasmid | $V_H$ gene | $J_H$ gene | HCDR3 length | $V_H$ mutated | light chain type | $V_L$ plasmid | $V_L$ gene | $J_L$ gene | LCDR3 length | $V_L$ mutated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T001 | total B | IgA1 | pH006176 | 1~3*01 | 3*02 | 12 | 3.13% | lambda | pL002315 | 2~23*01, 03 | 3*01 | 10 | 1.95% |
| T002 | total B | IgA1 | pH006175 | 3~7*01 | 4*02 | 9 | 4.28% | kappa | pK004590 | 2D~29*01 | 5*01 | 9 | 1.64% |
| T003 | mem B | IgG1 | pH006174 | 1~3*01 | 4*02 | 20 | 1.72% | kappa | pK004589 | 3~11*01 | 3*01 | 9 | 0.57% |
| T004 | total B | IgM | pH006177 | 3~53*01,02 | 4*02 | 17 | 2.28% | kappa | pK004591 | 3~20*01 | 1*01 | 10 | 1.12% |
| T005 | mem B | IgG1 | pH006250 | 7~4-1*02 | 5*02 | 10 | 6.10% | lambda | pL002355 | 2~14*03 | 3*02 | 10 | 3.87% |
| T006 | mem B | IgG1 | pH006246 | 4~30-4*01 | 4*02 | 11 | 4.18% | kappa | **pK004629** | 1~33*01 | 2*01, 02 | 9 | 4.57% |
| T007 | mem B | IgG3 | pH006251 | 1~69*06 | 6*03 | 25 | 6.16% | kappa | **pK004629** | 1~33*01 | 2*01, 02 | 9 | 4.57% |
| T008 | mem B | IgG3 | pH006251 | 1~69*06 | 6*03 | 25 | 6.16% | lambda | pL002357 | 1~47*02 | 3*02 | 11 | 4.19% |
| T009 | mem B | IgA1 | pH006247 | 3~23*01 | 4*02 | 14 | 4.11% | kappa | pK004630 | 3/OR2~268*02 | 4*01 | 9 | 3.40% |
| T010 | mem B | IgA1 | pH006247 | 3~23*01 | 4*02 | 14 | 4.11% | lambda | pL002352 | 3~21*02 | 1*01 | 11 | 5.31% |

**Table I. Antibody characteristics.** For recombinant antibody T009 (recAb T009), the original $V_L$ sequence (K004631, not shown) was identical to K004629 (shown in bold) at the amino acid level; K004629 was used for mAb production.

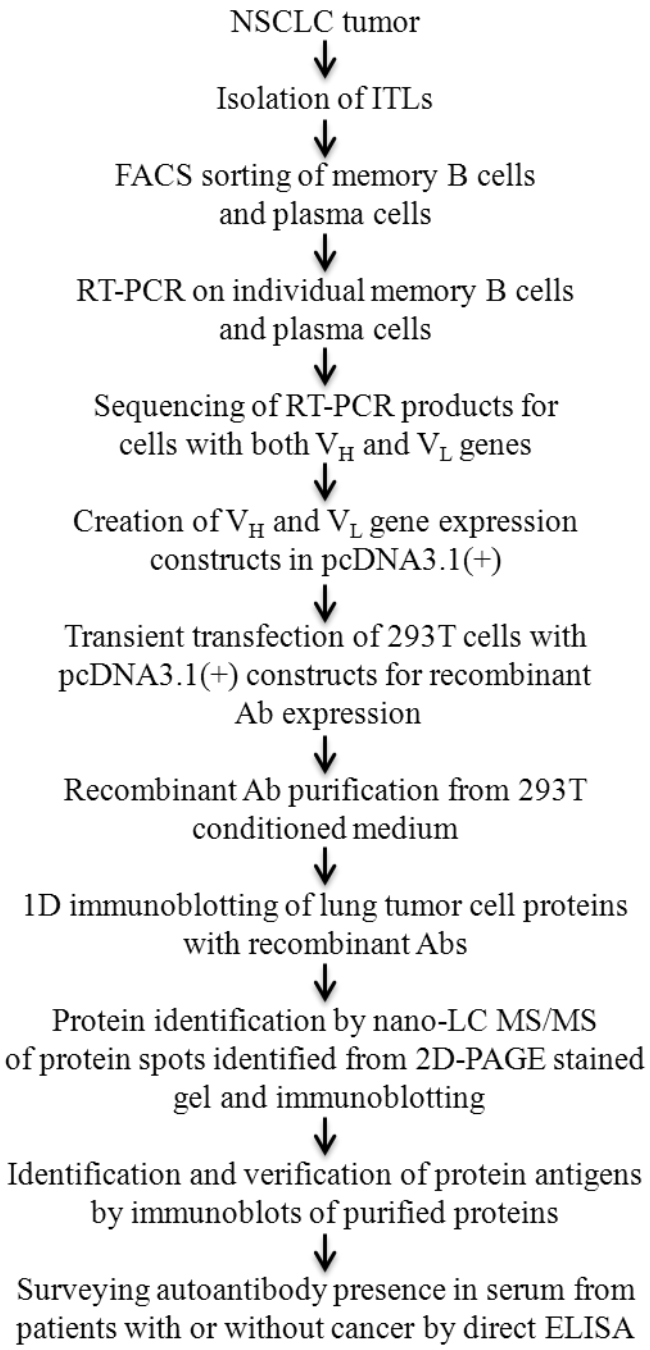



**FIGURE 1.** Work flow diagram.

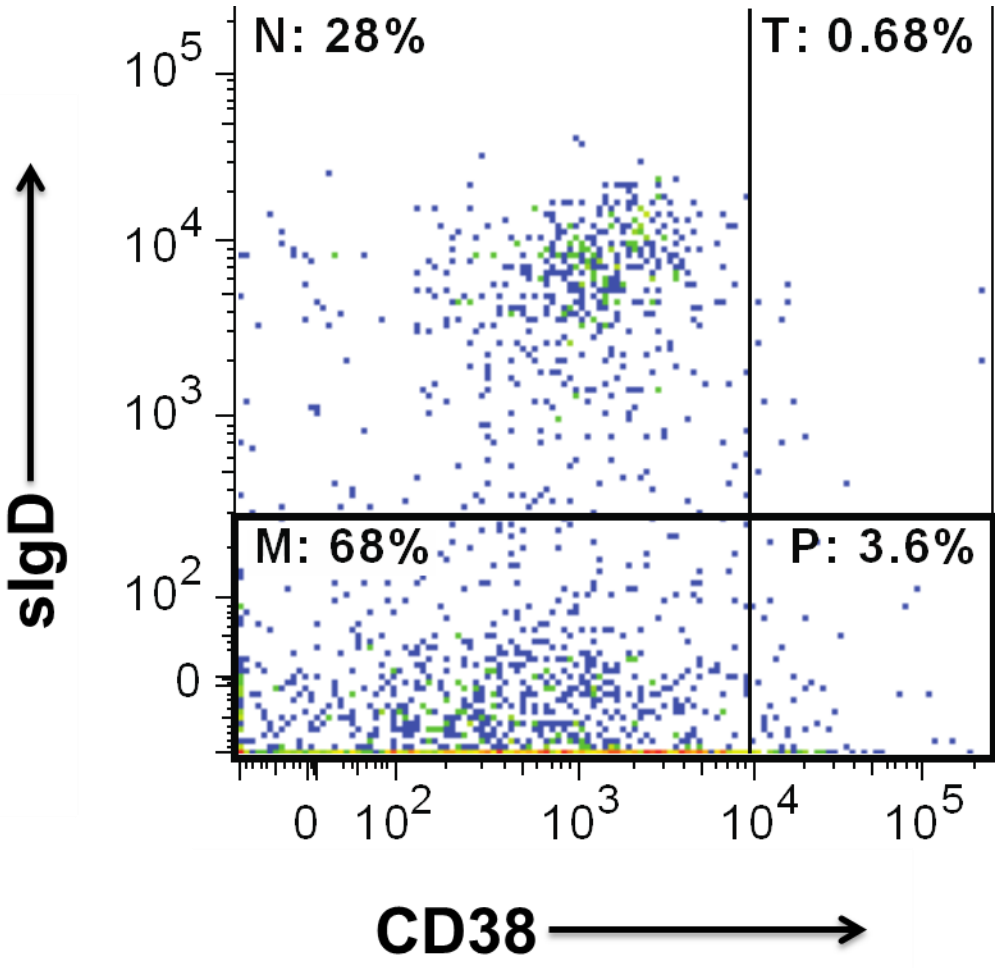



**FIGURE 2.** B cell populations among ITLs isolated from a lung tumor. ITLs were sorted as described in Materials and Methods. The percentages in each quadrant refer to the proportions of naïve (N), transitional (T) and memory B cells (M), and plasmablasts (P). The image was generated with FlowJo (Tree Star, Ashland, OR).

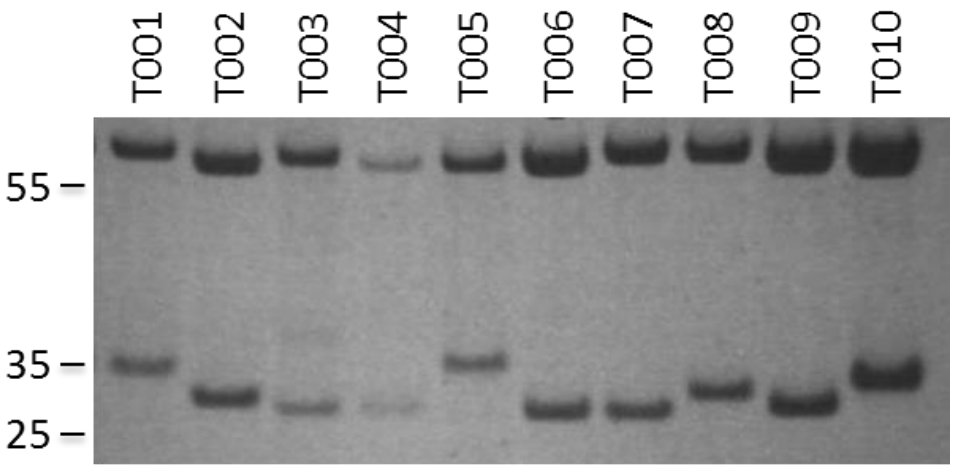
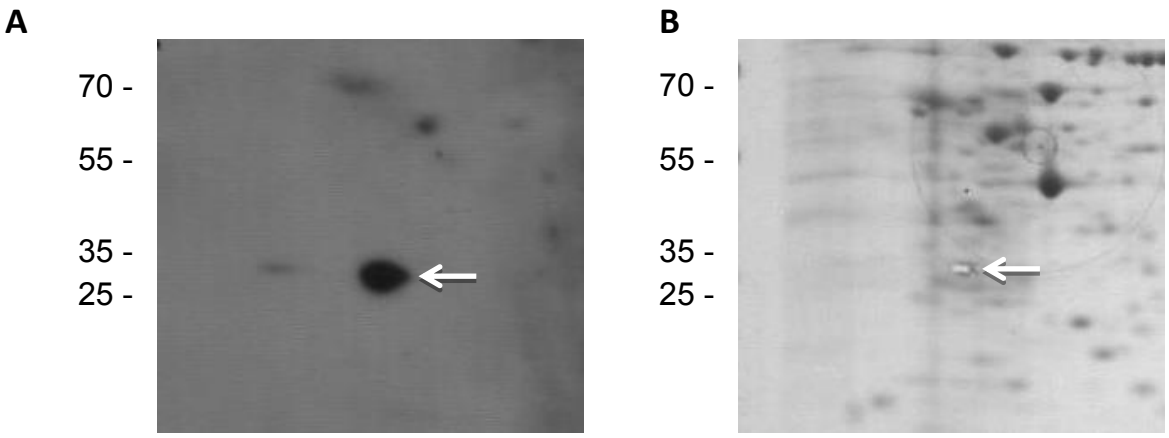



**FIGURE 3.** SDS-PAGE of purified recombinant antibodies. Recombinant antibodies, purified from 293T cell culture medium by protein G affinity chromatography, were analyzed via SDS-PAGE and silver staining. Well T004 contains 0.5 µg protein; all other wells contain 1 µg protein. Molecular weight markers, in kDa, are shown to the left.




**C**

TPM4_HUMAN (100%), 28,522.4 Da
Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3
8 unique peptides, 8 unique spectra, 8 total spectra, 69/248 amino acids (28% coverage)

MAGLNSLEAV KRKIQALQQQ ADEAEDRAQG LQRELDGERE RREK**AEGDVA**
**ALNR**R**IQLVE EELDR**AQER**L ATALQK**LEEA EKAADESERG MKVIENRAMK
DEEK**MEIQEM QLK**EAK**HIAE EADRKYEEVA R**K**LVILEGEL ER**AEERAEVS
ELKCGDLEEE LKNVTNNLKS LEAASEKYSE KEDKYEEEIK LLSDKLKEAE
TRAEFAERTV AKLEK**TIDDL EEK**LAQAKEE NVGLHQTLDQ TLNELNCI

**FIGURE 4.** Identification of the immunoreactive 2D gel spot as TPM4. Proteins from the lung cancer cell line H460 were separated on duplicate 2D gels. One gel was blotted to PVDF and probed with recombinant antibody T009 and the other gel was stained with Coomassie blue. The immunoreactive spot on the PVDF (arrow in A) was aligned with a spot on the stained gel, which was excised (arrow in B) and subjected to in-gel trypsin digestion and nano-LC MS/MS. All eight peptides identified from the MS analysis (bold underlined text in C) mapped to TPM4. Molecular weight markers, in kDa, are shown to the left.

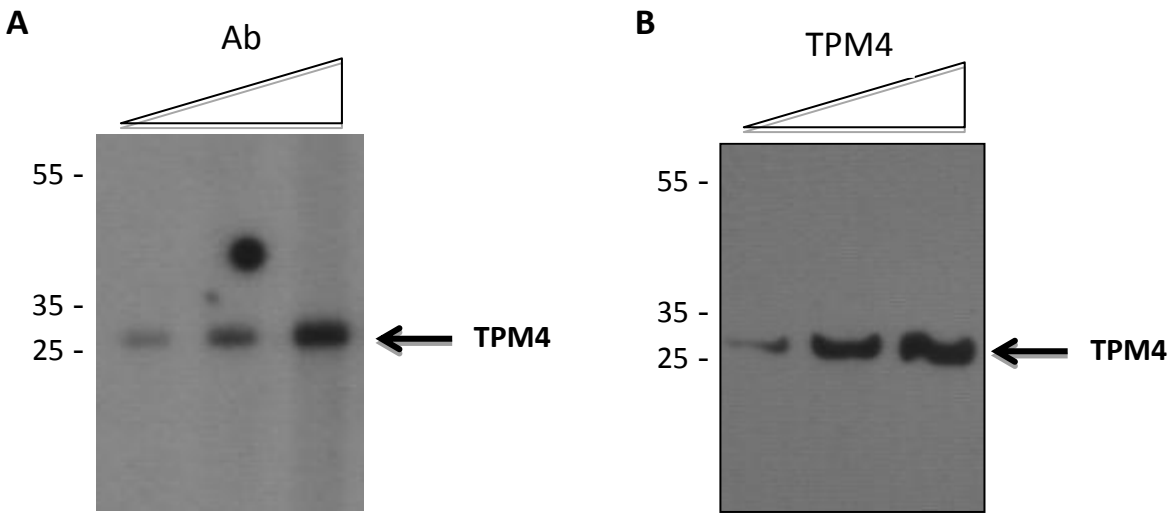



**FIGURE 5.** Verification of TPM4 as the T009 antigen. A, The recombinant antibody designated T009 was diluted to 0.625, 1.25, and 2.5 µg/ml and used to probe a blot containing 0.1 µg recombinant TPM4. Incubation of T009 with the blot was carried out in a Surf-Blot apparatus to allow different antibody concentrations to be run at the same time. B, Serum (1:1600) from the same patient who supplied the ITLs was used to probe a blot containing 0.25, 0.5, and 1 µg recombinant TPM4. Bound antibody was detected with anti-human IgG-gamma chain-HRP secondary antibody and chemiluminescent substrate. The recombinant TPM4 runs at a slightly higher molecular weight than native TPM4 due to the presence of an affinity tag.

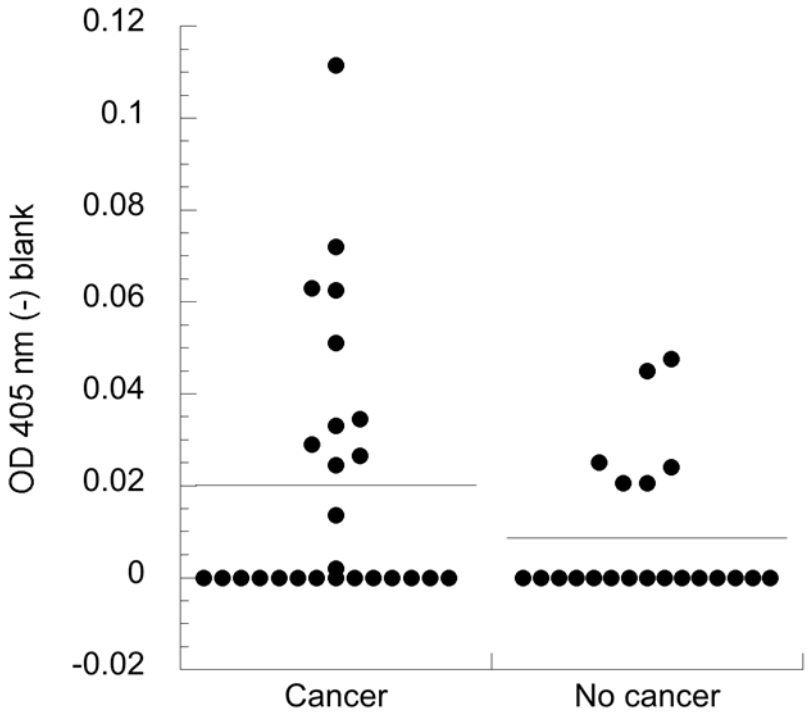



**FIGURE 6.** Prevalence of anti-TPM4 antibodies in lung cancer patients and controls. Serum from 26 patients with lung cancer and 21 without were tested by ELISA against immobilized recombinant TPM4. All sera were diluted 1:50. Optical density values at 405 nm from wells containing only blocking agent were subtracted from those with TPM4 plus blocking agent. All negative blank-corrected values are reported in the plot as zero. The horizontal lines show the average blank-corrected values.